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Stimulation of Local Immunity and Protection in Mice by Intramuscular Immunization with Triple- or Double-Layered Rotavirus Particles and QS-21

Monica M. McNeal,* Mary N. Rae,* Margaret E. Conner,† and Richard L. Ward*,[1]

*Division of Infectious Diseases, Children's Hospital Medical Center, Cincinnati, Ohio 45229; and †Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030

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Based on studies in animal models, parenteral immunization has become recognized as a potential vaccination strategy against rotavirus. Using an adult mouse model, the effects of the saponin adjuvant QS-21 on protection against murine rotavirus (strain EDIM) infection was determined following two intramuscular (i.m.) immunizations with purified EDIM particles including triple-layered (tl) infectious particles, tl particles inactivated with psoralen/UV, and double-layered (dl) inactivated particles. All three particles stimulated large serum rotavirus IgG responses and small amounts of serum rotavirus IgA, but undetectable stool rotavirus IgA. Inclusion of QS-21 during immunization increased the serum responses approximately 2- to 10-fold and also stimulated low levels of stool rotavirus IgA. Protection based on reduced shedding of rotavirus following EDIM challenge was significant ($P < 0.001$) with each immunized group and was enhanced ($P < 0.001$) by inclusion of QS-21 during immunization. Mice immunized with either live or inactivated tl particles and QS-21 were almost fully protected. Furthermore, animals inoculated with dl particles and the adjuvant shed significantly ($P = .02$) less virus following challenge than mice immunized with inactivated tl particles even though the latter induced measurable titers of neutralizing antibody to EDIM. These results demonstrate significant protection against rotavirus following i.m. immunization with both dl and tl EDIM particles which is consistently enhanced with QS-21.

INTRODUCTION

Rotavirus is the primary cause of severe diarrhea in infants and young children worldwide. Several vaccine candidates have been evaluated, all of which are live, orally deliverable, attenuated rotaviruses. These vaccines are intended to mimic the route of infection and subsequent protection associated with natural infection. However, rotavirus vaccine candidates tested to date have provided only partial protection, even against severe rotavirus disease (DeMol *et al.,* 1986; Christy *et al.,* 1988; Bernstein *et al.,* 1991, 1995; Santosham *et al.,* 1991; Clark *et al.,* 1995; Rennels *et al.,* 1996; Lanata *et al.,* 1996). Therefore, improved vaccines are clearly needed.

To facilitate the development of such vaccines and, at the same time, to gain an understanding of the mechanisms of active immunity against rotaviruses, an adult mouse model was developed in our laboratory (Ward *et al.,* 1990). Using this model, it was found that protection after oral immunization with live homologous or heterologous rotaviruses correlated with titers of serum and stool rotavirus IgA (McNeal *et al.,* 1994; Feng *et al.,* 1994), but no correlation was found between rotavirus IgG or serotype-specific neutralizing antibody titers and protection (Ward *et al.,* 1992). Further, it has been shown using genetically engineered, antibody deficient mice that complete protection against subsequent rotavirus infection was dependent on the presence of antibody (McNeal *et al.,* 1995; Franco and Greenberg, 1995). Finally, in a study using a hybridoma back-pack mouse model, it was found that protection from murine rotavirus challenge was obtained in mice inoculated with anti-VP6 IgA-secreting hybridoma cells, and no protection was obtained with anti-VP6 IgG-secreting hybridomas (Burns *et al.,* 1996). Thus, induction of serum or mucosal rotavirus IgA may be an important correlate of protection against infection with this intestinal pathogen.

Because protection against rotavirus disease was incomplete following oral immunization with candidate vaccines, alternative methods of immunization are being investigated. One method is parenteral immunization with live or inactivated rotavirus particles. Using the adult mouse model, we demonstrated that parenteral (i.e., intraperitoneal) immunization with live or UV-inactivated rotaviruses stimulated protection against challenge with the murine rotavirus strain EDIM (McNeal *et al.,* 1992). Although high titers of serum rotavirus IgG were induced, little or no serum or stool rotavirus IgA was detected in the immunized mice. This suggested that rotavirus IgG rather than IgA may be the better correlate of protection following parenteral immunization. Subsequently, Con-

[1] To whom correspondence and reprint requests should be addressed at Division of Infectious Diseases, Children's Hospital Medical Center, 3333 Burnet Avenue, CH-1, Cincinnati, OH 45229-3039. Fax: (513) 636-7682.

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ner *et al.* (1993) observed that two intramuscular immunizations of rabbits with live or formalin-inactivated simian rotavirus (strain SA11) provided complete protection against infection with a rabbit rotavirus. Again, no intestinal rotavirus IgA was found in the immunized rabbit, but instead, protection correlated with the presence of intestinal rotavirus IgG. Thus, in both studies of parenteral immunization in animal models, protection was associated with the presence of rotavirus IgG rather than IgA.

The purpose of the present study was to determine whether protection following parenteral immunization in the adult mouse model could be enhanced by the addition of an adjuvant, and whether the adjuvant would stimulate production of serum and intestinal rotavirus IgA. The saponin QS-21, purified from the bark of the *Quillaja saponaria* (Kensil *et al.,* 1991), has been shown to enhance antibody and cell mediated immune responses to a number of different antigens (Newman *et al.,* 1992; Hancock *et al.,* 1995, 1996). QS-21 has also been shown to modulate the isotype and even the IgG class of the antibody response to an antigen (Karagouni and Hadjipetrou-Kouvounakis, 1990; Kensil *et al.,* 1991; Kensil, 1996). Furthermore, QS-21 has been used in phase 1 trials in humans (Livingston *et al.,* 1994) and, therefore, may be a candidate for use in human vaccines. In this study, we determined whether the protection stimulated by intramuscular immunization with either live or inactivated purified EDIM virus particles could be enhanced by inclusion of QS-21.

RESULTS

Rotavirus particles used for immunization

Triple-layered (tl) EDIM virus particles purified by CsCl gradient centrifugation had a final titer of 8.4×10^8 focus-forming units (FFU)/ml while the double-layered (dl) EDIM particles obtained from the same CsCl gradient and subsequently treated with 10 mM EDTA before being repurified in CsCl had a titer of 3×10^3 FFU/ml. Based on protein concentrations in both preparations, the infectivity of the tl particles was 10^5 times greater than that of the dl particles. Following treatment with UV/psoralen, neither preparation had detectable infectious virus (i.e., $<10^2$ FFU/ml). Both tl (live and inactivated) and dl particles were analyzed by SDS–PAGE gel electrophoresis (Fig. 1). The tl particles contained the known structural proteins VP1, VP2, VP3, VP4, VP6, and VP7 while outer capsid proteins VP4 and VP7 were undetectable in dl particles.

Antibody responses following immunization

BALB/c mice were immunized by two i.m. injections with 20 μg of one of the three preparations of purified EDIM particles described above either with or without QS-21. Serum and stool samples collected 4 weeks after the second immunization but prior to challenge were examined for the presence of rotavirus antibody. All immunized groups of mice had very large and consistent serum rotavirus IgG responses (Table 1). Groups that received QS-21 with the EDIM particles had approximately 10-fold ($P < 0.001$) higher titers of rotavirus IgG than mice immunized with the same particles without adjuvant. Modest amounts of serum rotavirus IgA were also detected in each of the immunized groups (Table 1), and inclusion of QS-21 with the viral particles also resulted in significantly ($P < 0.001$) higher quantities of this antibody. No detectable stool rotavirus IgA was found in the groups immunized with the three different viral particles without QS-21 (Table 1). However, when QS-21 was included with any of these particles, significant ($P < 0.001$) levels of rotavirus stool IgA were detected. Thus, use of QS-21 as an adjuvant induced mucosal (intestinal) IgA responses following intramuscular inoculation.

Serum neutralizing antibody against EDIM was also found in all groups of mice immunized with the tl particles (Table 1). Infectious tl particles induced significantly ($P < 0.001$) more neutralizing antibody compared to inactivated particles. When QS-21 was included with the infectious particles, significantly ($P < 0.001$) more neutralizing antibody was induced. A small but insignificant ($P = 0.09$) increase in neutralizing antibody was also induced by inclusion of QS-21 with the inactivated tl particles. No neutralizing antibody was found in mice

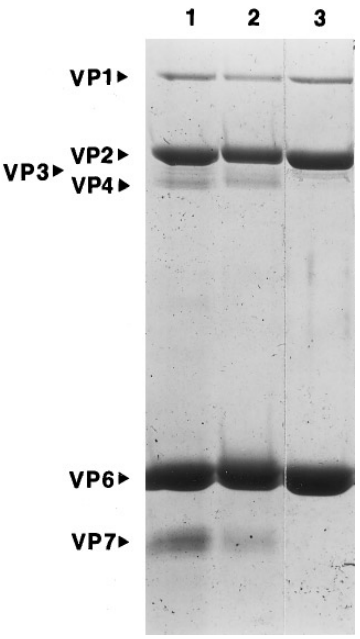

FIG. 1. SDS–polyacrylamide gel electrophoresis of purified EDIM particles. The rotavirus particles were purified as described under Materials and Methods and their proteins were dissociated and separated by electrophoresis in SDS–polyacrylamide gels. Protein bands were visualized by staining with Coomassie brilliant blue. Lane 1, ÷ infectious tl EDIM particles; lane 2, ÷ UV/psoralen inactivated tl particles; lane 3, ÷ psoralen/UV inactivated dl particles.

TABLE 1

Geometric Mean Titers of Rotavirus Antibodies in Mice at the Time of EDIM Challenge[a]

Group	Serum IgG (range)	Serum IgA (range)	Stool IgA (range)	Neutralizing antibody (range)
Unimmunized control	<100 (<100)	<100 (<100)	<5 (<5)	<20 (<20)
Triple-layered EDIM	1,570,375 (1,022,800–2,694,000)	414 (271–606)	<5 (<5)	330[b] (248–524)
Triple-layered EDIM +QS-21	12,898,880[c] (9,816,000–13,296,000)	785[c] (543–934)	134[c] (64–390)	1021[c] (640–1,560)
Inactivated triple-layered EDIM	1,206,889 (866,100–1,641,000)	332 (183–617)	<5 (<5)	62 (20–144)
Inactivated triple-layered EDIM +QS-21	9,203,958[c] (6,299,000–15,038,000)	897[c] (652–1,171)	116[c] (30–238)	283 (22–2,440)
Inactivated double-layered EDIM	1,051,074 (734,900–1,517,000)	267 (237–335)	<5 (<5)	<20 (<20)
Inactivated double-layered EDIM +QS-21	10,572,660[c] (9,305,000–12,626,000)	826[c] (522–1,440)	246[c] (61–622)	<20 (<20)

[a] Mice were immunized i.m. with 20 μg of viral particles with and without QS-21 on days 0 and 14. Blood and stool specimens collected 4 weeks after the second immunization but prior to EDIM challenge were used for this analysis.
[b] Significantly ($P < 0.001$) greater than the group inoculated with inactivated tl particles.
[c] Significantly ($P < 0.001$) greater than the group without QS-21.

inoculated with the inactivated dl particles (Table 1). This was consistent with the finding that VP4 and VP7 were not found after purification of these particles.

To further establish that VP4 and VP7 proteins were not present on dl particles, sera from mice immunized with the three different preparations of EDIM particles were used to immunoprecipitate ^{35}S-labeled EDIM proteins from infected cell lysates (Fig. 2). Antibodies against both structural and nonstructural rotavirus proteins were detectable in mice inoculated with infectious tl particles. This result demonstrates that at least limited replication of EDIM occurred following i.m. inoculation. Sera from mice inoculated with inactivated tl particles immunoprecipitated structural proteins but not the nonstructural proteins, further establishing that these viral particles were inactivated. The serum from animals immunized with the dl particles also precipitated structural proteins other than VP4 and VP7. The faint but detectable protein band immunoprecipitated with this serum that was observed at the position of VP7 was also found in comparable amounts after immunoprecipitation of the ^{35}S-labeled uninfected cell lysate (Fig. 2). These results support previous evidence that dl particles were free of the two outer capsid proteins.

As previously noted, the specific infectivity of purified tl particles before UV/psoralen treatment was 10^5 times greater than that of purified dl particles. Since the presence of the outer capsid VP4 and VP7 proteins is generally considered to be required for rotavirus infectivity, the small amount of residual infectivity in the dl particle preparation suggested that small quantities of VP4 and VP7 may still be present. However, it was also possible

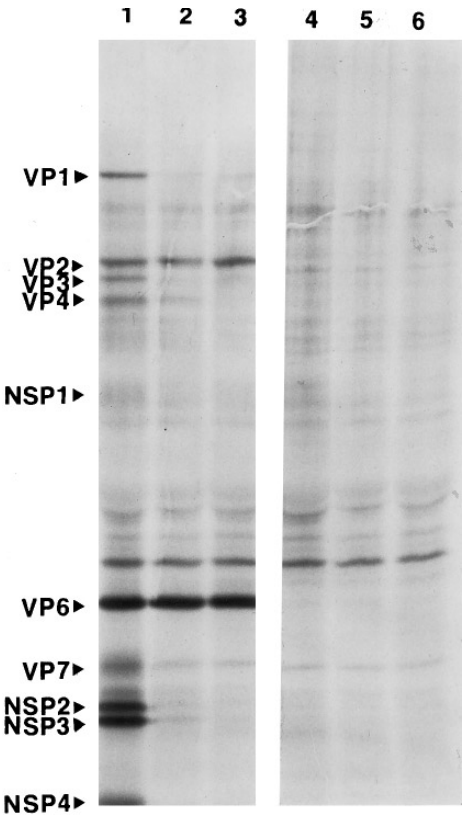

FIG. 2. SDS–polyacrylamide gel electrophoresis of ^{35}S-labeled proteins immunoprecipitated from EDIM-infected (lanes 1–3) or mock infectious tl particles (lanes 1 and 4); inactivated tl particles (lanes 2 and 5); or inactivated dl particles (lanes 3 and 6). Structural (VP) and nonstructural (NSP) protein assignments are based on their positions relative to molecular weight markers as described by Estes (1996).

TABLE 2

Effect of mAb 8H2 on the Infectivities of dl and tl Particle Preparations[a]

Treatment	Recoverable infectious virus (FFU/ml)	
	dl particles	tl particles
None	2.9×10^3	2.0×10^3
10^2 dilution of 8H2	$<3 \times 10^1$	1.9×10^3
10^3 dilution of 8H2	$<3 \times 10^1$	1.3×10^3
10^4 dilution of 8H2	1.6×10^3	1.5×10^3

[a]Purified preparations of dl and tl EDIM particles were appropriately diluted and incubated (30 min, 37°C) with an equal volume of mouse ascites fluid containing mAb 8H2 diluted between 100- and 10,000-fold. Recoverable infectivities (FFU/ml) were then determined and compared to those obtained without mAb treatment.

that dl particles retain some limited infectivity without VP4 and VP7. VP4 and VP7 are the rotavirus neutralization proteins and antibodies to either can neutralize rotavirus. Therefore, it was reasoned that if the dl particles retain some VP4 and VP7, their residual infectivity should be neutralized by treatment with murine hyperimmune serum to tl EDIM particles. When tested, it was found that such serum did, in fact, neutralize this residual infectivity (results not shown). However, this hyperimmune serum would also contain large quantities of anti-VP6 antibody which may have been responsible for dl particle neutralization. To determine if antibodies to VP6 could have this specific effect on dl but not tl particles, both particles were treated with an ascites preparation of mAb 8H2 which had been shown to be directed against an epitope on VP6 (Ginn *et al.,* 1992). Incubation with mAb 8H2 neutralized the residual infectivity of dl particles but had no effect on the infectivity of tl particles (Table 2). This result indicated that the residual infectivity in the dl EDIM preparation was due to particles that were specifically neutralized by anti-VP6 antibody and, therefore, was not due to fully encapsidated tl particles.

Protection following intramuscular immunization

Four weeks after the second immunization, mice were orally challenged with EDIM to determine the level of protection after i.m. immunization. Mice immunized with any of the viral particle preparations were significantly protected as compared to the control (unimmunized) mice based on the mean days of viral shedding and the mean quantity of virus shed per mouse (Table 3). Protection was even found in mice inoculated with dl particles where no neutralizing antibody was generated. Inclusion of QS-21 had dramatic effects on the levels of protection achieved with all three virus preparations. Complete protection was obtained with infectious tl particles, nearly complete protection was stimulated by inactivated tl particles (only one mouse shed low amounts of rotavirus for 3 days), and only three of the six mice shed rotavirus for 2–4 days in mice immunized with dl particles containing QS-21. When only the mean quantity of virus shed was compared, infectious tl particles without adjuvant stimulated significantly ($P < 0.001$) greater protection than either inactivated tl particles or dl particles. Inactivated tl particles also provided greater protection than dl particles ($P = 0.05$). It should be noted that inclusion of QS-21 with the dl particles stimulated significantly ($P = 0.02$)

TABLE 3

Shedding of Rotavirus Antigen after EDIM Challenge[a]

Group	*N*	Number shedding EDIM	Mean days of viral shedding [b]	Mean quantity of antigen shed[c]
Unimmunized control	6	6	6.8	1.68
Triple-layered EDIM	6	5	3.1[d]	0.28[d,e]
Triple-layered EDIM +QS-21	6	0	0[d,f]	0[d,f]
Inactivated triple-layered EDIM	6	5	3.8[d]	0.67[d,g]
Inactivated triple-layered EDIM +QS-21	6	1	0.5[d,f]	0.02[d,f]
Inactivated double-layered EDIM	6	6	4.5[d]	1.17[h]
Inactivated double-layered EDIM +QS-21	6	3	1.7[d,i]	0.13[d,f]

[a] Mice were challenged with 4×10^4 FFU of EDIM. Stools were collected from each mouse for 10 days and tested for the presence of rotavirus antigen.
[b] Defined by the total number of days shedding per animal per group.
[c] Quantity defined by average A_{490} values above background for every mouse on each of the 10 days after challenge as determined by ELISA.
[d] Significantly ($P < 0.001$) less than unimmunized control group.
[e] Significantly ($P < 0.001$) less than group receiving inactivated tl or dl particles.
[f] Significantly ($P < 0.001$) less than group without QS-21.
[g] Significantly ($P = 0.05$) less than group receiving dl particles.
[h] Significantly ($P < 0.01$) less than unimmunized control group.
[i] Significantly ($P < 0.01$) less than group without QS-21.

TABLE 4

Geometric Mean Titers of Postchallenge Rotavirus Antibodies in Mice That Shed EDIM[a]

Group	Number that shed EDIM	Serum IgG (range)	Serum IgA (range)	Stool IgA (range)
Unimmunized control	6	125,866 (98,477–155,806)	7,793 (4,950–13,814)	1,442 (993–1,963)
Triple-layered EDIM	5	1,810,991[b] (1,245,600–2,592,000)	42,560[b] (32,765–53,022)	3,699[c] (2,433–6,356)
Triple-layered EDIM +QS-21	0	—	—	—
Inactivated triple-layered EDIM	5	1,836,137[b] (1,554,800–1,989,300)	65,580[b] (47,562–82,692)	4,038 (512–17,669)
Inactivated triple-layered EDIM +QS-21	1	9,502,000	50,083	7,521
Inactivated double-layered EDIM	6	1,842,466[b] (1,729,000–2,079,000)	32,236[b] (20,403–56,086)	3,042[d] (1,501–4,629)
Inactivated double-layered EDIM +QS-21	3	7,861,457[b] (7,624,000–8,300,000)	17,952[b] (16,981–19,355)	2,688 (961–4,559

[a] Stool and blood specimens were collected 21 days after challenge with EDIM. All groups contained 6 mice. Values are the GMT of only those animals that shed detectable levels of antigen.

[b] Significantly ($P < 0.001$) greater than unimmunized control group.

[c] Significantly ($P < 0.002$) greater than unimmunized control group.

[d] Significantly ($P < 0.01$) greater than unimmunized control group.

better protection than was obtained with inactivated tl particles without adjuvant.

Rotavirus antibody responses after challenge

To determine the effects of immunization on postchallenge antibody titers, serum and stool specimens collected 21 days after EDIM challenge were analyzed for rotavirus antibody. All unimmunized mice developed large serum rotavirus IgG responses, but the immunized animals all had extremely high IgG titers before challenge, and even those that shed rotavirus after challenge developed insignificant (i.e. <fourfold) rises in this antibody (Tables 1 and 4). In contrast, there was excellent agreement between shedding in immunized mice following challenge and significant (≥fourfold) increases in both serum and stool rotavirus IgA titers; i.e., only one immunized mouse that failed to shed virus developed a significant stool rotavirus IgA response, and one immunized mouse that shed virus failed to develop a fourfold increase in stool rotavirus IgA. It was of interest to note that even though mice immunized without QS-21 developed no detectable stool rotavirus IgA, these mice were apparently primed to produce this antibody because two of the three groups developed significantly ($P \leq .01$) higher titers following challenge than the unimmunized group (Table 4).

DISCUSSION

Rotavirus vaccine candidates that have been evaluated in human trials have all been live, attenuated virus strains that are delivered orally to mimic natural infection. The protection provided has been inconsistent, even against severe disease. Parenteral rotavirus vaccines were initially discounted because rotavirus is a disease of the intestinal mucosa, and intestinal immune responses, which develop primarily after antigen exposure to mucosal lymphoid tissues, were believed to be required for protection. This was supported by studies in lambs (Snodgrass and Wells, 1976) and mice (Offit and Clark, 1985) which indicated that intestinal, but not circulating rotavirus antibody, was protective against rotavirus disease. We subsequently reported, however, that intraperitoneal immunization with infectious or UV inactivated rotaviruses provided good protection against rotavirus infection in adult mice (McNeal *et al.,* 1992). Conner *et al.* (1993) also found that intramuscular immunization of rabbits with infectious or inactivated simian rotavirus protected against subsequent rabbit rotavirus infection. Therefore, parenteral immunization has become recognized as a possible supplement or alternative to oral immunization.

Almost all parenteral vaccines are delivered by intramuscular inoculation, a route found to provide only partial protection of mice against murine rotavirus infection in the absence of adjuvant. We, therefore, determined whether this protection could be significantly augmented if adjuvant was included in the immunization protocol. The adjuvant chosen was QS-21 because of its low toxicity and established ability to enhance both antibody and cell mediated immune responses.

Mice immunized by two intramuscular injections with purified, infectious or inactivated tl murine rotavirus (EDIM) particles or dl particles lacking outer capsid proteins VP4 and VP7 shed less virus than naive mice when

orally challenged with EDIM four weeks after the second immunization. Addition of QS-21 to the immunization regimen consistently enhanced this protection irrespective of which EDIM particles were used as the immunogen. Intramuscular immunization also consistently induced large serum rotavirus IgG responses, and inclusion of QS-21 increased these responses approximately 10-fold. In spite of these very large responses, however, protection was only complete in mice immunized with infectious tl particles plus QS-21.

It was previously reported that protection following oral immunization of mice with live virus correlated with higher titers of both serum and stool rotavirus IgA (McNeal *et al.,* 1994; Feng *et al.,* 1994). Following intramuscular inoculation with EDIM particles, low levels of serum rotavirus IgA were stimulated by each of the virus particles, and these increased approximately threefold when QS-21 was included ($P < 0.001$ for each group). Therefore, the increased protection associated with QS-21 also correlated with small but consistent increases in serum rotavirus IgA. The association, however, was most dramatic when stool rotavirus IgA titers were examined. In this case, no responses were found following immunization in the absence of adjuvant, and low but consistent stool rotavirus IgA titers were found in all mice when QS-21 was included with each immunogen. Even with adjuvant, however, these titers were still approximately 10-fold less than generated by oral immunization with live EDIM (see Tables 1 and 4). Since oral immunization stimulated long-lasting serum and intestinal rotavirus antibody responses and complete protection for at least 14 months (McNeal and Ward, 1995), it would be of interest to determine the duration of both rotavirus antibody and protection following i.m. immunization.

Because three types of rotavirus particles were used in this study, it was of interest to compare the antibody responses and protection induced by each. The infectious tl particles stimulated antibody production against both structural and non-structural rotavirus proteins (see Fig. 2) implying that the virus at least initiated a replication cycle when injected intramuscularly. However, this did not result in larger serum rotavirus IgG or IgA titers or more stool rotavirus IgA but did cause an increased level of neutralizing antibody to EDIM (see Table 1). The protection provided by infectious EDIM particles was no greater than with inactivated EDIM when immunization was performed without adjuvant. Additional mice would be required to determine whether protection was significantly greater using the infectious particles in the presence of QS-21. Regardless of whether or not the infectious particles provided greater protection than inactivated EDIM, this protection was not due to intestinal replication. Had this occurred, the rotavirus IgA responses would have been comparable to those found after oral inoculation of unimmunized mice with live EDIM.

The other immunogen examined was dl EDIM particles lacking the VP4 and VP7 outer capsid proteins. These particles stimulated comparable titers of serum rotavirus IgG and IgA, as well as stool rotavirus IgA, relative to tl particles, probably because the inner capsid protein VP6 is the dominant immunogen. Since VP4 and VP7 are the neutralization proteins, however, the dl particles stimulated no detectable neutralizing antibody. Likewise, the antibody they stimulated immunoprecipitated only inner capsid proteins (see Fig. 2). Even so, these particles elicited protection against EDIM, and when injected i.m. with QS-21, the dl particles provided significantly ($P = 0.02$) greater protection against shedding of EDIM than inactivated tl particles injected without adjuvant. The lack of dependence on neutralizing antibody for protection supports previous conclusions found with this model, i.e. mice were either orally or intraperitoneally immunized with heterotypic rotaviruses, made no neutralizing antibody to EDIM, and were still significantly protected against shedding of EDIM when challenged (Ward *et al.,* 1992; McNeal *et al.,* 1992). Evidence that immune responses to neither VP4 nor VP7 are required for protection is supported by two recent reports. The first was that virus-like particles lacking these proteins stimulate protection when delivered either orally or intranasally (O'Neal *et al.,* 1997); the second was that injection of mice with hybridoma cells that produced IgA against VP6 protected against rotavirus infection (Burns *et al.,* 1996). Although these results indicate that neither VP4 or VP7 are required to stimulate immunity in this model, protection was consistently enhanced when these proteins were present. Whether this enhancement was due to stimulation of neutralizing antibody by epitopes on these proteins or possibly to production of other immunological effectors such as non-neutralizing antibody on cytotoxic T lymphocytes remains to be determined.

A study published over a decade ago reported that mAbs against VP6 had neutralizing activity against rotavirus (Sabara *et al.,* 1987). Because VP4 and VP7 are the recognized rotavirus neutralization proteins, this report has been largely ignored. The finding of low level, residual infectivity in the dl particle preparation, however, suggested the possibility that such particles may be neutralized by antibody to VP6. To test this possibility, we attempted to neutralize both tl and dl particles with an anti-VP6 mAb and found it neutralized only the dl particles (see Table 2). The conclusions from this observation are that the infectivity in the dl preparation is due to particles lacking VP4 and VP7 and that such particles can be neutralized with an anti-VP6 antibody. The mechanism by which this neutralization occurs remains to be determined.

The powerful adjuvant effects exerted by QS-21 in this study could be due to a number of mechanisms. Saponin adjuvants such as QS-21 have been shown to stimulate the

immune system by increasing both T_H1 and T_H2 responses (Karagouni *et al.,* 1990; Bomford *et al.,* 1992; Valensi *et al.,* 1994; Maloy *et al.,* 1995). Regarding antibody production, T_H1 responses augment IgG2a production while T_H2 responses favor the production of IgG1 and IgA. QS-21 has been shown to induce the three major subclasses of the IgG isotype: G1, G2a and G2b (Kensil *et al.,* 1991). Although intestinal rotavirus IgG has been associated with protection in rabbits (Conner *et al.,* 1993), we were able to detect only very low titers (GMT of <30 units/ml for any mouse group) of stool rotavirus IgG in mice administered any EDIM particle, even in association with QS-21. Since inclusion of this adjuvant increased the levels of serum rotavirus IgG and protection, it is possible that a mechanism of increased protection induced by QS-21 in this model was through stimulation of extremely high titers of serum rotavirus IgG.

Another possible protective mechanism for QS-21 is stimulation of T_H2 responses and, subsequently, rotavirus IgA levels. The effect of QS-21 on IgA was most pronounced in stool specimens where titers increased from undetectable (i.e., <5 units/ml) to as high as 622 units/ml. Because the ratios between these titers and those of serum rotavirus IgA did not correlate on an animal-by-animal basis, it appeared that the presence of rotavirus IgA in stool was not due merely to increased concentrations of serum rotavirus IgA. To our knowledge, this is the first direct evidence that QS-21 can induce intestinal IgA following parenteral immunization. In a study by Thaper *et al.* (1991) using ISCOMS containing saponins, it was shown that a vaginal mucosal response was induced after i.m. immunization. In addition, studies by Ahmeida *et al.* (1992, 1993) showed that influenza virus given by i.m. inoculation with ISCOMS induced nasal IgA responses comparable to that found after intranasal immunization with live virus, and the i.m. immunization was as protective as the nasal immunization. Finally, using a passive protection baboon model, Snodgrass *et al.* (1995) reported an increase in rotavirus IgA, IgG, and neutralizing antibody in milk following intramuscular injection of pregnant females with rotavirus and ISCOMS suggesting a boosting of a mucosal response. Although the components of ISCOMS responsible for these mucosal immune responses were not identified, it may have been saponins such as QS-21.

The results presented in this study demonstrate that active immunity to rotavirus can be obtained following intramuscular injection with live or inactivated tl EDIM particles or with dl particles lacking VP4 and VP7. The addition of the saponin adjuvant QS-21 increased the level of protection obtained with each viral particle. Since rotavirus serum IgG and IgA and stool IgA were all dramatically increased by the addition of QS-21, it cannot be predicted which, if any, was responsible for the higher level of protection. If induction of mucosal rotavirus IgA, which has been found to correlate with protection following oral immunization with live rotavirus, is necessary for protection, QS-21 acting as an immune modulator may be an excellent adjuvant to include with other parenteral rotavirus vaccines.

MATERIALS AND METHODS

Mice

Female BALB/c mice purchased from Harlan–Sprague–Dawley (Indianapolis, IN) were used at 6 weeks of age. All animals were found to have no serum rotavirus IgG or IgA or stool rotavirus IgA as determined by ELISA (McNeal *et al.,* 1995). Microisolator cages were used to house the animals during the entire study. Six animals were used in each group (three/cage). All procedures were conducted in accordance with protocols approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee.

Virus and viral particles for immunization

The G3 [P16] EDIM strain of rotavirus used in this study was adapted to grow in cell culture by passage in MA104 cells as previously described (Ward *et al.,* 1990). A preparation of the ninth cell culture passage containing 2×10^6 FFU per ml was used to orally challenge mice. The EDIM virus used to generate viral particles for immunization was a triply plaque picked isolate that had been passaged a total of 41 times in MA104 cells and purified by CsCl centrifugation (McNeal *et al.,* 1992). Tl infectious virus (1.36 g/ml) and dl particles lacking VP4 and VP7 (1.38 g/ml) were collected from the same gradient. The dl particles were further treated with 10 mM EDTA and purified a second time in CsCl to ensure complete removal of VP4 and VP7 proteins. Following purification, the virus particles were dialyzed against phosphate-buffered saline containing 20% glycerol and stored frozen at $-70°C$. To establish which proteins were present in the particles, each was analyzed by SDS–polyacrylamide gel electrophoresis as previously described (McNeal *et al.,* 1992). Purified tl or dl EDIM particles were inactivated by UV/psoralen treatment as described by Groene and Shaw (1992). In short, the particles were dispensed into 60-mm petri dishes and psoralen (4′-aminomethyltroxsalen HCl; Lee Biomolecular Research, Inc., San Diego, CA) was added at a concentration of 40 μg/ml. The virus was then placed in ice on a rotating platform at a distance of 10 cm and irradiated with high intensity, longwave length UV light (365 nm, 40 min). Following treatment, the viral titer was determined by a fluorescent focus assay as previously described (Knowlton *et al.,* 1991). Although the infectivity of tl particles containing 8.4×10^8 FFU/ml was reduced below detectable limits (i.e., 10^2 FFU/ml) by 20 min of UV treatment, the irradiation time was extended to 40 min to ensure that no infectious virus remained.

Monoclonal antibody (mAb)

Ascites fluid obtained from mice inoculated with a hybridoma producing mAb 8H2/G5 was used to neutralize purified tl and dl EDIM particles prior to UV/psoralen treatment using procedures already described (Knowlton *et al.,* 1991). This mAb was shown to be specific for rotavirus protein VP6 (Ginn *et al.,* 1992).

Study design

Mice were immunized by i.m. injection with viral particles including tl infectious virus, tl inactivated virus, or dl inactivated virus. Each injection was with 50 μl containing 20 μg of viral protein, either with or without 20 μg of QS-21 (Wyeth-Lederle Vaccines and Pediatrics, Pearl River, NY), into the thigh muscle of the hind leg. Two immunizations were given 2 weeks apart beginning on day 0. Mice were then orally challenged with 4×10^4 FFU of EDIM 4 weeks after the last injection. Stool specimens were collected daily for 10 days after challenge and analyzed for rotavirus antigen. Blood specimens were collected by retroorbital capillary plexus puncture on day 0, on the day before challenge, and 21 days after challenge. Stool specimens were also collected at these time points. These blood and stool specimens were used to measure rotavirus serum IgA, IgG and neutralizing antibody, and rotavirus stool IgA titers.

Detection of rotavirus antigen in stool

Two stool pellets collected into 0.5 ml of Earle's balanced salt solution (EBSS) on each day following EDIM challenge were stored frozen (−20°C). To test for rotavirus antigen, the specimens were thawed, homogenized, and analyzed by ELISA as previously described (McNeal *et al.,* 1994). The quantity of virus shed was determined by net A_{490} values (i.e., the average of two rotavirus negative antibody-coated wells subtracted from the average of two rotavirus antibody-coated wells). A_{490} values of $\geq$3.0 were assigned values of 4.0 for quantitation purposes. To determine the amount of rotavirus shed per mouse within a group, the average A_{490} values above background were determined for every mouse on each of the 10 days after EDIM challenge.

Determination of rotavirus antibody titers

Serum rotavirus IgA and IgG and rotavirus stool IgA were measured by ELISA as previously described (McNeal *et al.,* 1995). Briefly, EIA plates (Corning Costar Co., Cambridge, MA) were coated overnight at 4°C with anti-rotavirus rabbit IgG. After washing with phosphate buffered saline plus 0.05% Tween 20, EDIM viral lysate and mock-infected cell lysate were each added to duplicate wells. Serial 2-fold dilutions of pooled sera from EDIM infected mice assigned concentrations of 160,000 or 10,000 units/ml of rotavirus IgG or IgA, respectively, were added to duplicate wells coated with either EDIM-infected or uninfected MA104 cell lysates to generate a standard curve. Serial 10-fold dilutions of mouse sera to be tested were also added to duplicate wells of each lysate. This was followed by sequential addition of biotin-conjugated goat anti-mouse IgG or IgA (Sigma Chemical Co., St. Louis, MO), peroxidase-conjugated avidin-biotin (Vector Laboratories, Burlingame, CA), and *o*-phenylenediamine substrate (Sigma Chemical Co.). Color development was stopped after 15 min with 1 M H_2SO_4 and the A_{490} was measured. Titers of rotavirus IgG or IgA, expressed as units/ml, were determined from the standard curve generated by subtraction of the average A_{490} values of the duplicate cell lysate wells from the average of the wells coated with EDIM lysate.

For determination of stool rotavirus IgA, two stool pellets were collected into 0.5 ml of EBSS, homogenized, and centrifuged (1500*g,* 5 min). Stool rotavirus IgA was then measured by the method described above.

Neutralizing antibody to EDIM was determined by using an antigen reduction ELISA assay as described previously (Knowlton *et al.,* 1991).

Immunoprecipitation of viral proteins

Pooled sera from mice inoculated with tl or dl particles (live or inactivated) were used to immunoprecipitate ^{35}S-labeled EDIM to identify immunogenic EDIM proteins. The procedures for radiolabeling EDIM, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis have been previously described (McNeal *et al.,* 1992).

Statistical analyses

Differences in the mean quantity of rotavirus antigen shed per mouse and the mean number of days shedding between groups were determined by Student's *t* test. Differences in geometric mean titers of rotavirus antibody between groups of mice were evaluated by this same statistical test.

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